APPENDIX 1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:		§	
		§	
Iris Pecker et al.		§	
		§	
Serial No.:	09/988,113	§	
		§	
Filed:	November 19, 2001	§	Group Art Unit: 1652
		§	
For:	POLYNUCLEOTIDE ENCODING A POLYPEPTIDE HAVING HEPARANASE ACTIVITY AND EXPRESSION OF SAME IN GENETICALLY MODIFIED CELLS	§ § § § §	Attorney Docket: 01/22781
		§	
Examiner: R. HUTSON		§	

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

DECLARATION OF IRIS PECKER UNDER 37 CFR 1.132

I am presently employed as researcher in Insight Strategy & Marketing Ltd. I received my Ph.D. in molecular biology degree from the Hebrew University of Jerusalem (Israel) in 1994, after which I worked as a post-doctoral fellow in the Tel Aviv University (Israel) in the field of Human Genetics. In 1996 I joined Insight and presently serve as the head of the molecular biology department, supervising the activities of 10 other workers.

I am a co-inventor of the subject matter claimed in the above-referenced U.S. patent application.

I have read the Examiner's Office Action dated November 19, 2001. I hereby declare the following:

Heparanase has a specific, well characterized and unique catalytic activity known for over 20 years. Over the years, heparanase was partially purified from a variety of mammalian sources. Heparanase is defined as a GAG hydrolase which

cleaves heparin and heparan sulfate (both are sulfated) at the beta-1,4 linkage between glucuronic acid and glucosamine. Heparanase is an endolytic enzyme and the average product length it generates is 8-12 saccharides.

The present invention represents a significant, non-obvious, inventive advance over the prior art.

The present invention encompasses polynucleotides encoding for polypeptides having a sequence of at least 70% homology to SEQ ID NO:10. Many different heparanase sequences are known, having different degrees of homology to the sequence of SEQ ID NO:10, which is a human sequence. For example, mouse B16-F10 heparanase as well as human platelet heparanases and heparanase enzymes produced by several human tumor cell lines are known. However, the mouse heparanase amino acid sequence is known to have less than 80% identity to human heparanase, as described in published PCT Application No. WO 00/52178: POLYNUCLEOTIDE ENCODING A POLYPEPTIDE HAVING HEPARANASE ACTIVITY AND EXPRESSION OF SAME IN GENETICALLY MODIFIED CELLS. Furthermore, sequence information available about a variant of the B16-F10 cell line shows that the sequence of heparanase in the cell line is apparently identical to the sequence of heparanase in normal mouse tissue. This example was already described in the present Application, for example in Figure 17. Therefore, actual experimental support (in the form of examples) is provided for heparanase sequences that have homology of less than about 80%.

To further clarify this point, Applicant has submitted alignment data in the attached Appendix, showing the homology (and differences) between human, rat, mouse and chicken heparanase sequences (part of this information has already been included in the present Application; see for example Figure 17). Some important

shared feanures such as active site residues and the heparin binding sites are marked. This information further supports Applicant's statements with regard to both the heparanase sequences of the present invention, and also the ability of one of ordinary skill in the art to readily recognize a heparanase protein as such.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United states Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

August 12, 2003

Iris Pecker

Dr. Iris Pecker

APPENDIX - Sequence Homology Data

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                    10        20        30        40        50        60
                     |         |         |         |         |         |
mouse       -MLR-------LLLLWLWGPLGALAQGAPAGTAPTDDVVDLEFYTKRPLRSVSPSFLSIT
rat         -MLRP------LLLLWLWGRLRALTQGTPAGTAPTKDVVDLEFYTKRLFQSVSPSFLSIT
human       MLLRSKPALPPPLMLLLLGPLGPLSPGALPRPAQAQDVVDLDFFTQEPLHLVSPSFLSVT
chicken     ----------MLVLLLLVLLLAVPP--------RR-TAELQLGLREPIGAVSPAFLSLT
                       *:* *   * .:.           ..:*::  :. :  ***:***:*

                    70        80        90       100       110       120
                     |         |         |         |         |         |
mouse       IDASLATDPRFLTFLGSPRLRALARGLSPAYLRFGGTKTDFLIFDPDKEPTSEERSYWKS
rat         IDASLATDPRFLTFLGSPRLRALARGLSPAYLRFGGTKTDFLIFDPNKEPTSEERSYWQS
human       IDANLATDPRFLILLGSPKLRTLARGLSPAYLRFGGTKTDFLIFDPKKEGTFEERSYWQS
chicken     LDASLARDPRFVALLRHPKLHTLASGLSPGFLRFGGTSTDFLIFNPNKDSTWEEKVLSEF
            :**.** ****: :*  *:*::** ****.:******.******:*.*:.* **:   :

                   130       140       150       160       170       180
                     |         |         |         |         |         |
mouse       QVNHDICRSEPVSAAVLRKLQVEWPFQELLLLREQYQKEFKNSTYSRSSVDMLYSFAKCS
rat         QDNNDICGSERVSADVLRKLQMEWPFQELLLLREQYQREFKNSTYSRSSVDMLYSFAKCS
human       QVNQDICKYGSIPPDVLEKLRLEWPYQEQLLLREHYQKKFKNGTYSRSSVDVLYTFANCS
chicken     QAK-DVCEAWPSFAVVPKLLLTQWPLQEKLLLAEHSMKKHKNTTITRSTLDILHTFASGS
            * : *:*      . * . *  :** ** *** *: .:.:**.* :**::*:*::**..*

                   190       200       210       220       230       240
                     |         |         |         |         |         |
mouse       GLDLIFGLNALLRTPDLRWNSSNAQLLLDYCSSKGYNISWELGNEPNSFWKKAHILIDGL
rat         RLDLIFGLNALLRTPDLRWNSSNAQLLLNYCSSKGYNISWELGNEPNSFWKKAQISIDGL
human       GLDLIFGLNALLRTADLQWNSSNAQLLLDYCSSKGYNISWELGNEPNSFLKKADIFINGS
chicken     CFRLVFGLNALLRRAGLQWDSSNAKQLLGYCAQRSYNISWELGNEPNSFRKKSGICIDGF
             : *:******** ..*:*:****: **.**:.:.************** **: * *:*

                   250       260       270       280       290       300
                     |         |         |         |         |         |
mouse       QLGEDFVELHKLLQRS-AFQNAKLYGPDIGQPRGKTVKLLRSFLKAGGEVIDSLTWHHYY
rat         QLGEDFVELHKLLQKS-AFQNAKLYGPDIGQPRGKTVKLLRSFLKAGGEVIDSLTWHHYY
human       QLGEDYIQLHKLLRKS-TFKNAKLYGPDVGQPRRKTAKLLKSFLKAGGEVIDSVTWHHYY
chicken     QLGRDFVHLRQLLSQHPLYRHAELYGLDVGQPRKHTQHLLRSFMKSGGKAIDSVTWHHYY
            ***.*::.*::** :   :::*:*** *:**** :* ::*:**:*:**:.***:******

                   310       320       330       340       350       360
                     |         |         |         |         |         |
mouse       LNGRIATKEDFLSSDALDTFILSVQKILKVTKEITPGKKVWLGETSSAYGGGAPLLSNTF
rat         LNGRVATKEDFLSSDVLDTFILSVQKILKVTKEMTPGKKVWLGETSSAYGGGAPLLSNTF
human       LNGRTATREDFLNPDVLDIFISSVQKVFQVVESTRPGKKVWLGETSSAYGGGAPLLSDTF
chicken     VNGRSATREDFLSPEVLDSFATAIHDVLGIVEATVPGKKVWLGETGSAYGGGAPQLSNTY
            :*** **:****..:.** *  ::::.:: :.:   **********.******** **:*:

                   370       380       390       400       410       420
                     |         |         |         |         |         |
mouse       AAGFMWLDKLGLSAQMGIEVVMRQVFFGAGNYHLVDENFEPLPDYWLGLLFKKLVGPRVL
rat         AAGFMWLDKLGLSAQLGIEVVMRQVFFGAGNYHLVDENFEPLPDYWLSLLFKKLVGPKVL
human       AAGFMWLDKLGLSARMGIEVVMRQVFFGAGNYHLVDENFDPLPDYWLSLLFKKLVGTKVL
chicken     VAGFMWLDKLGLAARRGIDVVMRQVSFGAGSYHLVDAGFKPLPDYWLSLLYKRLVGTRVL
            .**********:*: **:******* **** .***** .*.**********:*:***.:**

                   430       440       450       460       470       480
                     |         |         |         |         |         |
mouse       LSRVKGPDRSKLRVYLHCTNVYHPRYQEGDLTLYVLNLHNVTKHLKVPPPLFRKPVDTYL
rat         MSRVKGPDRSKLRVYLHCTNVYHPRYREGDLTLYVLNLHNVTKHLKLPPPMFSRPVDKYL
human       MASVQGSKRRKLRVYLHCTNTDNPRYKEGDLTLYAINLHNVTKYLRLPYPFSNKQVDKYL
chicken     QASVEQADARRPRVYLHCTNPRHPKYREGDVTLFALNLSNVTQSLQLPKQLWSKSVDQYL
             : *: ..  : *********  :*:*:***:**:.:** ***: *::*  :  : ** **

                   490       500       510       520       530       540
                     |         |         |         |         |         |
mouse       LKPSCPDCLLSKSVQLNCQILKMVDEQTLPALTEKPLPAGSALSLPAFSYGFFVIRNAKI
rat         LKPFGSDGLLSKSVQLNGQTLKMVDEQTLPALTEKPLPAGSSLSVPAFSYGFFVIRNAKI
human       LRPLGPHGLLSKSVQLNGLTLKMVDDQTLPPLMEKPLRPGSSLGLPAFSYSFFVIRNAKV
chicken     LLPHGKDSILSREVQLNGRLLQMVDDETLPALHEMALAPGSTLGLPAFSYGFYVIRNAKA
            * * * ..:**:.***** *:***::***.* * .* .**:*.:***** .*:******

mouse       AACI
rat         AACI
human       AACI
chicken     IACI
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Multiple alignment of heparanase from Human, Rat, Mouse and chicken generated by Clustal W. Active site residues are bolded and putative heparin binding sites are boxed.

APPENDIX 2

DECLARATION OF ISRAEL VLODAVSKY

I am presently employed as researcher in the Hadassah-Hebrew University Hospital (Department of Oncology, Jeruasalem 91120, Israel). I chair the Tumor Biology Research Unit of the Sharett Oncology Institute of the Hadassah Hospital. I am a full professor in the Faculty of Medicine of the Hebrew University School of Medicine. I received my Ph.D degree from the Weizmann Institute of Science (Rehovot, Israel) in 1975, worked as a post-doctoral fellow in UCLA and UCSF and was a visiting Professor at Harvard Medical School (Children's Hospital, Boston). In 1980 I established a Tumor Biology Research Laboratory at the Hadassah-University Hospital. My research focuses on basic and clinical aspects of tumor metastasis and angiogenesis, with emphasis on cell interaction with the extracellular matrix, heparin-binding growth factors and heparin/heparan sulfate-degrading enzymes. Since the beginning of my carrier, I have published 247 scientific articles in highly regarded journals and books, and have presented my achievements at more than 90 international scientific conferences. I am a member of several international scientific societies and important local committees, and was awarded the 1997 prize for a distinguished Israeli scientist in medicine. For the last 20 years I have been engaged in the research of heparanase and heparan sulfate and have published over 70 papers in the field (see enclosed curriculum vitae).

I hereby declare the following:

Heparanase has a specific, well characterized and unique catalytic activity known for over 20 years. Over the years, heparanase was partially purified from a variety of mammalian sources. Heparanase is defined as a GAG hydrolase which cleaves heparin and heparan sulfate (both are sulfated) at the b1,4 linkage between glucuronic acid and glucosamin. Heparanase is an endolytic enzyme and the avcrage

product length it generates is 8-12 saccharides. The other known heparin/heparan sulfate degrading enzymes are b-glucuronidase, a-L iduronidase and a-N acetylglucosaminidase. These three enzymes are exolytic enzymes, each of which cleaves a specific linkage within the polysaccharide chain and generates disaccharides. These issues are further addressed below.

There are three sources of reports regarding false heparanase antibodies as follows:

An anti PAI-1 antibody, which is described in U.S. Pat. No. 5,362,641 (D3), was produced in an attempt to elicit anti-heparanase antibodies. This antibody was elicited by a PAI-1 contamination in a purified sample of heparanase, as was observed by peptide analysis.

Identification of this antibody as an anti PAI-1 antibody is discussed in U.S. Patent No. 5,968,822. Page 11, line 18 to page 12, line 2, recite in this respect that:

> Several years ago we prepared rabbit polyclonal antibodies directed against our partially purified preparation of human placenta heparanase. These antibodies, referred to in U.S. Pat. No. 5,362,641, were later found to be directed against plasminogen activator inhibitor type I (PAI-1) that was co-purified with the placental heparanase. These findings led to a modification of the original purification protocol to remove the PAI-1 contaminant.

Another false anti-heparanase antibody was generated against the chemokine CTAP III, a protein that was reported to possess heparanase-like activity. These antibodies were generated by the group of Prof. Ledbetter as described in Hoogewerf et al. J Biol Chem 17;270(7):3268-77, 1995) and were donated to other researchers as reported by Kosir et al. (J Surg Res. 67(1):98-105, 1997, page 99, materials and methods, right column, western blot).

CTAP III is a low molecular weight chemokine, which has no homology to heparanase from human placenta, SK-hepatoma, platelets (Hullet et. al. Nat. Med

5(7): 803-809, 1999) and SV40 transformed fibroblasts (Toyoshima and Nakajima J. Biol. Chem. 274(34):24153-24160, 1999) which were all purified and cloned recently and correspond to the amino acid sequence set forth in SEQ ID NO. 2 of the present application.

Because CTAPIII and heparanase, as defined by SEQ ID NO:2 of the present application, share no sequence homology, these antibodies are irrelevant. In addition, it was declared and it is now accepted by the scientific community, as is recited below, that CTAPIII was erroneously thought to be heparanase.

The third false anti-heparanase antibody was generated in the laboratory of Prof. Nicolson and was first reported by Jin et al. (Int J Cancer. 45(6):1088-95, 1990). This group isolated a 96 kDa mouse protein and used a peptide derived from the N-terminus of the partially purified protein to generate polyclonal as well as monoclonal antibodies. These antibodies detect a 96 kDa protein, which is obviously different from the placental heparanase referred to in the instant application and which was later isolated from other tissues as currently reported by several other groups. These antibodies were used by several research groups in collaboration with either one of the authors of the original paper (Marchetti et al. Cancer Res. 56(12):2856-63, 1996, Marchetti and Nicolson, Adv Enzyme Regul. 37:111-34, 1997, Mollinedo et al. Biochem J. 327(3):917-23, 1997).

In 1994, Vouge et al. (Int. J. Cancer 56:286-294, 1994) pointed out the fact that the antibodies claimed to detect heparanase actually detect GR94/endoplasmin, a previously cloned and characterized murine heat shock protein. The sequence and the molecular weight were in perfect agreement with those reported for the 96 kDa murine heparanase isolated by Nicolson's group. Later on, the mis-identification of the heparanase enzyme and consequently the antibodies generated against it was

admitted and accepted by the scientific community. The late papers (1996, 1997) still referring to these antibodies, as heparanase specific, are obscure. There is no doubt, however, that those antibodies do not recognize heparanase.

Kosir et al. provided yet another source of confusion in the field of heparanase, which only now has been resolved. Kosir et al. disclose anti-CTAP III antibodies. CTAP III is a platelet derives chemokine to which heparanase activity was erroneously attributed in the past. No sequence homology is observed between CTAP III and heparanases derived from, for example, placenta and from hepatoma cells (SEQ ID NO:2 of the present application, Vlodavsky et al. Nat. Med. 5(7):793-802, 1999, Kosir et al. Biochem Biophys Res Commun. 261(1):183-7, 1999) and as was later reported, from platelets (Hullet et al. Nature Medicine 5(7):803-809, 1999) and from SV40 transformed fibroblasts (Toyoshima and Nakajima J. Biol. Chem. 274(34):24153-24160, 1999).

The antibody used by Kosir at al. (J Surg Res. 67(1):98-105, 1997) was donated thereto by Dr. Ledbetter. According to lines 15-16 of the "Western blot" section, production of these antibodies was described in Hoogewerf et al. (J. Biol. Chem. 270(7): 3268-77, 1995). In this paper Hoogwerf et al., a research group from Upjohn Company, Kalamazoo, Michigan, describe the identification of CXC chemokines (the CTAPIII family) as heparan sulfate degrading enzymes. The antibodies described in the paper were raised against CTAPIII, which shares no sequence homology with the 50 kDa heparanase. Moreover, in a recent paper the same group from Pharmacia and Upjohn, Inc. retracted from their earlier statement regarding the heparanase activity of CTAPIII (Fairbank et al. J Biol Chem 274(42): 29587-29590, 1999) page 29590, right column, last paragraph of the discussion. They state that:

> Finally, an earlier report from this laboratory suggested that heparanase was a post-translationally modified form of a CXC chemokine, namely CTAPIII (7). We have not been able to confirm this observation, nor have others who have purified and characterized human heparanase

In this paragraph they refer to their previous paper, Hoogewerf et al. (J Biol Chem 1995 Feb 17;270(7):3268-77).

It is accepted today by the scientific community that CTAPIII is not a heparanase or a precursor thereof.

I turn now to a detailed discussion of the references cited by the Examiner in the recent Official action and which are said to teach anti-heparanase antibodies.

As is evident from the background section of the instant application, the response filed herewith (see in particular the concluding remarks, and the arguments) and this declaration, the need for anti-heparanase antibodies is well recognized for many years and many unsuccessful attempts were made to obtain such antibodies. D3 clearly recognizes a particular need for anti-heparanase antibodies, however, recognizing a need does not qualify as anticipation. The need for anti-heparanase antibodies is indeed recognized by the art. However, this need was not fulfilled, nor does D3 fulfill this need.

Thus, D3 fails to teach anti-heparanase antibodies, and certainly does not teach or suggest the many important antibody assays described in the present application.

Marchetti et al. briefly mention the use of antibody developed against heparanase. No description of an antibody source, preparation or characteristics is provided. No data is shown regarding such an antibody. The authors refer to a manuscript in preparation. I failed to find any later publications which provide this data. In a similar paper published by Marchetti and Nicolson (Adv Enzyme Regul.

37:111-34, 1997) the same "results" are briefly described with the remark "data not shown" (see, page 127, the paragraph just before the discussion). In a more recent paper which discusses heparanase regulation in human melanoma and where Marchetti is the last author, the results are based solely on activity measurements (Walch et al. Int. J. Cancer 82:112-120, 1999). Mollinedo et al. (Biochem J. 327(3):917-23, 1997) report localization of heparanase using the monoclonal antibody and refer on page 918 (materials and methods, antibodies) to Marchetti at al. (Cancer Res. 56:2856-2863, 1996). Mollinedo et al. show immunoblots where the heparanase antibody detects a 96 kDa protein (page 920, Figure 2).

It is my knowledge that this antibody was generated in the laboratory of Prof. Nicolson and was first reported by Jin et al. (Int J Cancer. 45(6):1088-95, 1990). This group isolated a 96 kDa mouse protein and used a peptide derived from the N-terminus of the partially purified protein to generate polyclonal as well as monoclonal antibodies. These antibodies detect a 96 kDa protein, which is obviously different from placental heparanase and which was later isolated from other tissues as currently reported by several groups. These antibodies were used by several research groups in collaboration with either one of the authors of the original paper (Marchetti et al. Cancer Res. 56(12):2856-63, 1996, Marchetti and Nicolson, Adv Enzyme Regul. 37:111-34, 1997, Mollinedo et al. Biochem J. 327(3):917-23, 1997). In 1994, Vouge et al. (Int. J. Cancer 56:286-294, 1994) pointed out the fact that the antibodies claimed to detect heparanase actually detect GR94/endoplasmin, a previously cloned and characterized murine heat shock protein. The sequence and the molecular weight were in perfect agreement with those reported for the 96 kDa murine heparanase isolated by Nicolson's group. Later on, the mis-identification of the heparanase

enzyme and consequently the antibodies generated against it was admitted and accepted by the scientific community.

Interestingly, Prof. Nicolson has abandoned heparanase research and does not take part in the major progress achieved during the recent years. Dr. Nakajima is a researcher at Novartis, a company that published recently the cloning of heparanase, with Nakajima as a last author (Toyoshima and Nakajima, J. Biol. Chem. 274(34):24153-24160, 1999). The published sequence is identical to SEQ ID NO:2 listed in the present application and the molecular weight of the purified protein is of 50 kDa.

Thus, clearly these allegedly "anti-heparanase" antibodies were not specific for heparanase, unlike the antibodies of the present invention.

Thus, it is clear that the antibodies according to the present invention are novel and are also inventive over the background art, none of which taught or suggested a truly specific molecular probe against heparanase.

I hereby certify that the above facts and statements are true and complete, to the best of my knowledge.

Israel Vlodavsky

Prof. Israel Vlodavsky

Date: June 12, 2003

APPENDIX 3

DECLARATION OF IRIS PECKER

I, Iris Pecker, am the Head of Department of Molecular Biology at Insight Ltd. at Rehovot, Israel. I have a PhD in Molecular Biology, and received my degree from The Hebrew University of Jerusalem. My professional specialization is in gene and protein discovery and development.

I am an inventor of the present invention. I have read the present application and the new and amended claims, as well as the Office Action from the Examiner and the accompanying references. In support of the accompanying Response to this Office Action, I set forth below the results of experiments which were performed in my laboratory.

Briefly, the present invention relates to molecular probes which are specific to heparanase proteins. The specificity of these molecular probes would not have been obtained without a source of truly pure heparanase protein and also of the heparanase nucleotide sequence. Neither of these could have been obtained without cloning heparanase. Without such cloning, the nucleotide sequence would have remained unknown. Furthermore, as described in greater detail below, at the time of filing the present application, truly pure heparanase protein could only be obtained with recombinant heparanase.

The present invention represents a significant, non-obvious, inventive advance over the prior art such as Fuks (US Patent No. 5,362,641), which failed to

identify a sequence for heparanase, or Hoogwerf (PCT Application No. WO 95/04158), which failed to correctly identify a heparanase sequence. In fact, these two references illustrate many of the pitfalls for an inventor who wished to determine the amino acid sequence of heparanase at the time of filing the present application.

Although the description of the sequence of events which led to the determination of the amino acid sequence of heparanase appears to be quite straightforward, it does not include all of the many problems and obstacles that were overcome in order to clone heparanase. Heparanase was an unusually difficult protein to sequence, largely because identification of true heparanase was so problematic. Hoogwerf was one of many inventors who thought that they had obtained the true heparanase sequence, only to discover that a contaminant present in the "pure heparanase" had been sequenced instead. In the case of Hoogwerf, the sequenced contaminant turned out to be the CXC chemokine CTAP III, a low molecular weight chemokine which has no homology to heparanase, as described above. However, it could just as easily have been one of the many other contaminants which have been mistakenly identified as heparanase, such as PAI1, the protein against which Fuks mistakenly raised an "anti-heparanase" antibody. As described above, the antibody of Fuks was later determined to bind to PAI1 rather than heparanase. PAI1 has no heparanase activity, but is present in a significant amount in the protein mixture obtained after the purification procedure of Fuks.

Many different processes for purifying heparanase have been proposed, such as that of Fuks, yet all have a common result: production of a mixture with many significant protein components, only one of which is heparanase. Like Fuks, many different scientists have mistakenly identified various proteins as being heparanase; a partial list is provided in the attached Appendix B. Like Fuks, such a mistaken identification resulted at least in part from the belief that the particular purification process followed actually produced a pure heparanase.

I, as one of the inventors, also found that the purification process which we followed did not produce pure heparanase, but rather produced a mixture of proteins. Unlike previous scientists, however, we were able to overcome such a lack of purity by following a complex and difficult path to clone heparanase.

The purification process which we performed followed the teachings of Fuks, resulting in a mixture of proteins after the performance of Mono-S HPLC purification (col 16, lines 46-54 of Fuks). This mixture of proteins is described in the attached Appendix A. The mixture had heparanase activity, as in fact a portion of this mixture included heparanase itself. However, it also included other proteins, including PAI-1, a known contaminant of heparanase obtained through protein purification.

The performance of tryptic digestion and microsequencing of the mixture of proteins resulted in multiple protein (amino acid) sequences being obtained. We compared these sequences against the database of known proteins, and found that a number of such sequences did not match any known protein. These

sequences are given in Appendix C, with some exemplary identified sequences from known proteins. The presence of multiple unidentified sequences was a concern to us, as we knew that many previous "heparanase" sequences or purificd proteins had actually proven to be a contaminant of a purified heparanase, rather than heparanase itself.

We were able to select the correct short sequence obtained from microsequencing by using the database to identify a single clone. The sequence of this clone was used to isolate an extended cDNA and the clones were then assembled to form the correct sequence. However, highly surprisingly, a polypeptide was obtained which had 543 amino acids and a calculated molecular weight of 61,178 daltons. Such a finding was surprising because Fuks taught that the *expected* molecular weight of heparanase was 50 kD (col 15, lines 19-23 and 50-55).

We then decided to produce heparanase *(in cell culture)* from the cloned gene, in order to examine the activity of the gene product. as demonstrated in Appendix D. Attempting to transfect a mammalian cell line was not possible, because it would result in the problem of being unable to distinguish heparanase activity produced by the product of the transfected heparanase gene, and that of native heparanase, since the most commonly used mammalian cell lines have basal endogenous heparanase activity, as seen for example in the cell lines 293 and CHO (Chinese hamster ovary cells) . Indeed, as we later demonstrated had we transfected the cloned gene into bacteria which is thc most obvious cxpression

system or yeast, no activity would have been observed and we would have assumed that we had not obtained the true heparanase gene.

Bacteria, yeast and mammalian cell lines are standard expression systems for mammalian proteins such as heparanase. Failure to obtain active heparanase in the microbial systems is due to the lack of correct post translational modifications. Other cell expression systems, apart from mammalian cells, are usually not expected to be able to overcome this problem. These expression systems may also fail to produce a protein with the correct and apparently essential post-translational modifications, since such modifications differ between different cell systems. Only by choosing an unusual cell expression system, and transfecting insect cells with the heparanase gene sequence, using the Baculovirus expression system, were we able to demonstrate heparanase activity encoded by the isolated cDNA. Insect cells are not regularly used for mammalian proteins, and were certainly not the cell line of choice for the expression of a newly identified gene. Appendix E demonstrates why insect cells are not usually a cell line of choice for mammalian proteins, and also some of the typical uses for insect cells with the Baculovirus expression system for gene transfection.

As described in the present Application, the use of insect cells with the Baculovirus expression system results in the production of active heparanase gene product. However, the full length polypeptide for which the heparanase gene codes, which is about 66 kDa, is actually a prepro-heparanase form of the protein,

and has no heparanase activity. Instead, this form of the protein is cleavable to form a protein having heparanase activity.

A residual level of such activity can be detected due to the effect of non specific protease activity, which results in the activation of a minor fraction of the recombinant enzyme. We found that the detection of heparanase activity required several micrograms of recombinant enzyme, a quantity that is much higher than expected from a catalytic enzyme. This finding raised doubts about the functional identification of the cloned cDNA, and caused us to doubt whether the correct gene had been identified and cloned.

Although cleavage of the prepro-heparanase results in catalytic activity of the enzyme, attempts to obtain an actual heparanase protein through protein cleavage is likely to result in inactive protein. The activating protease is unknown and using non-specific proteases such as trypsin requires extensive studies in order to obtain an activated protein rather than a degraded protein. The pro-heparanase protein can be divided into three sections: an 8 kDa section, a 6 kDa section and the main 45 kDa section. In order to produce heparanase, the 6 kDa section is removed and the 8 kDa section joins to the 45 kDa section.

Thus, we were forced to overcome many barriers in order to obtain the sequence of heparanase, which was an unusually difficult protein to sequence even at the time of filing of the present application.

By overcoming these barriers, we were able to obtain novel and inventive molecular probes, such as specifically binding antibodies and specifically

hybridizable nucleic acid sequences, which could not have been previously obtained. One very important aspect of the present invention is its specificity, which could not have been obtained with previous "anti-heparanase" antibodies, as these antibodies were later found to cross-react with other proteins. Without the complete nucleic acid sequence, specifically hybridizable nucleic acid sequences could not have been obtained.

We also further developed these probes in order to obtain useful assays, which could never be accomplished by the background art. These useful assays, such as the detection of heparanase or some type of marker for heparanase (such as mRNA, for example) in body fluids, would not have been possible without the necessary tools, which are the specific molecular probes.

Another important issue is that certain of the above proteins which caused false anti-heparanase antibodies to be produced may themselves be contaminated with heparanase, explaining their misidentification as heparanase.. For example, commercially available preparations of CTAP III were shown to be contaminated with heparanase.

CTAP III is a low molecular weight chemokine, which has no homology to heparanase from human placenta, SK-hepatoma, platelets (Hullet et. al. Nat. Med 5(7): 803-809, 1999) and SV40 transformed fibroblasts (Toyoshima and Nakajima J. Biol. Chem. 274(34):24153-24160, 1999) which were all purified and cloned recently and correspond to the amino acid sequence set forth in SEQ ID NO. 2 of U.S. Patent No. 5,968,822 (Application No. 08/922,170).

Because CTAPIII and heparanase, as defined by SEQ ID NO:2 of the present Application, share no sequence homology, and no common epitopes, antibodies raised against CTAPIII are irrelevant to the present invention.

This point is further illustrated with experiments performed with CTAPIII in my laboratory. A commercial preparation of CTAP III (β-thromboglobulin) was purchased from CalBiochem (Cat. No. 605165). Five micrograms of CTAP III 1 ng and 0.5 ng of the mature form of recombinant heparanase (50 kDa or interchangeably 45 kDa) produced in CHO cells were electrophoresed through a poliacrylamide gel. Another gel was loaded with only 5 micrograms of CTAP III, was stained with Commassie blue and photographed (Figure 1B). A single band of about 6.5 kDa is apparent. The other gel was blotted onto nitrocellulose and the blot subjected to a Western blot analysis with an anti-heparanase polyclonal antibody, raised against SEQ ID NO:2 (this antibody is described in U.S. Patent No. 5,968,822 (Application No. 08/922,170) (Figure 1A).

The results clearly show that (i) the antibody is not cross reactive with CTAP III, as, in the right most lane no signal is evident close to the 6.5 kDa indication, although 5 micrograms of CTAP III are present there; and (ii) the CTAP III preparation analyzed is contaminated with both mature heparanase (50 kDa) and preheparanase (60 kDa). The amount of mature (catalytically active) heparanase present in 5 micrograms of CTAP III is estimated according to Figure 1A to be about 0.75 ng.

In Figure 2, the activity of heparanase present in 5 micrograms CTAP III was compared to 0.5 and 1 ng of pure recombinant mature heparanase, using a radiolabeled ECM degradation assay. The activity results are in full agreement with the Western blot results, indicating that the amount of active heparanase present in 5 micrograms of the CTAP III preparation is equivalent to between 0.5 ng and 1 ng active heparanase.

FIGURE 1

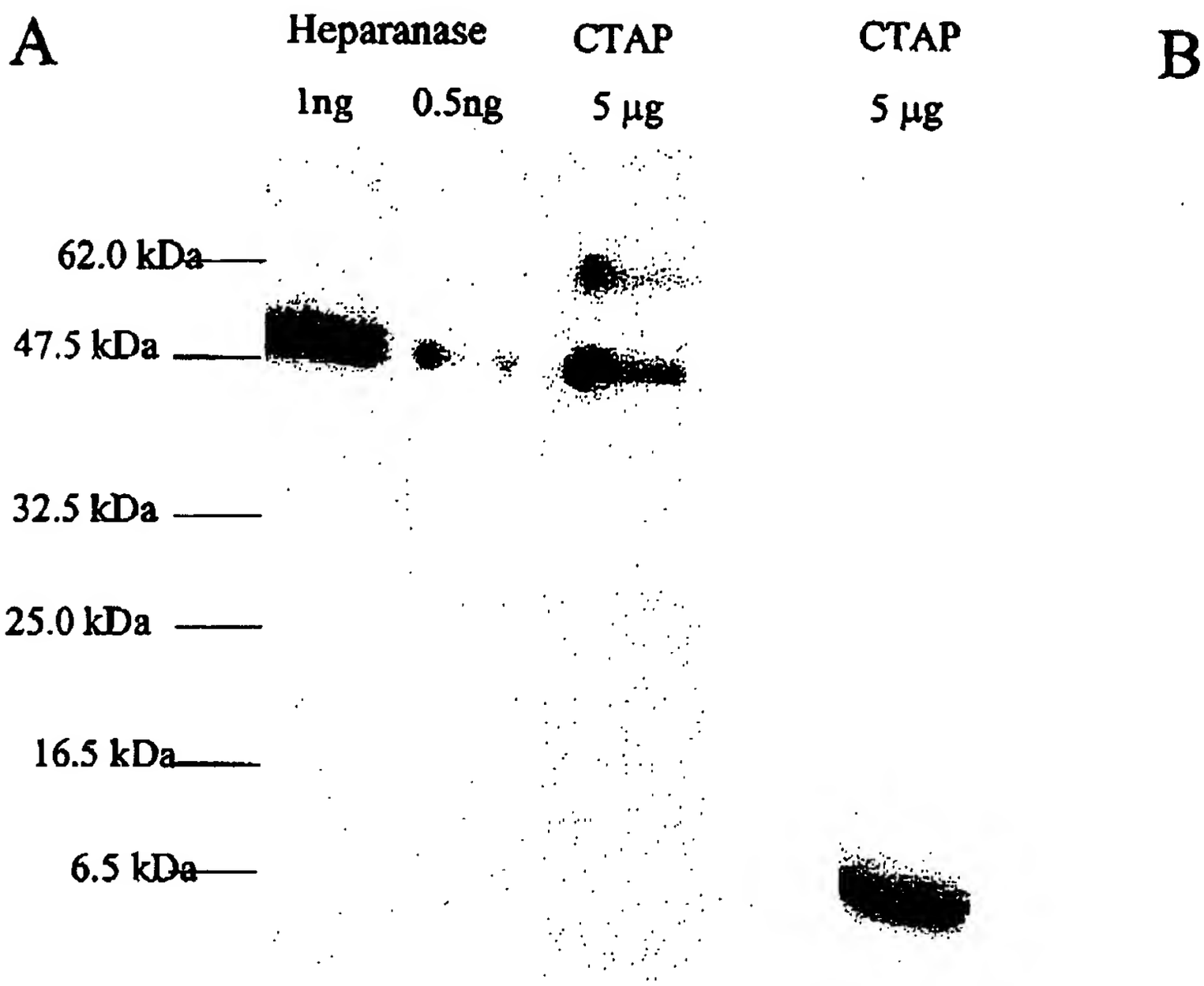
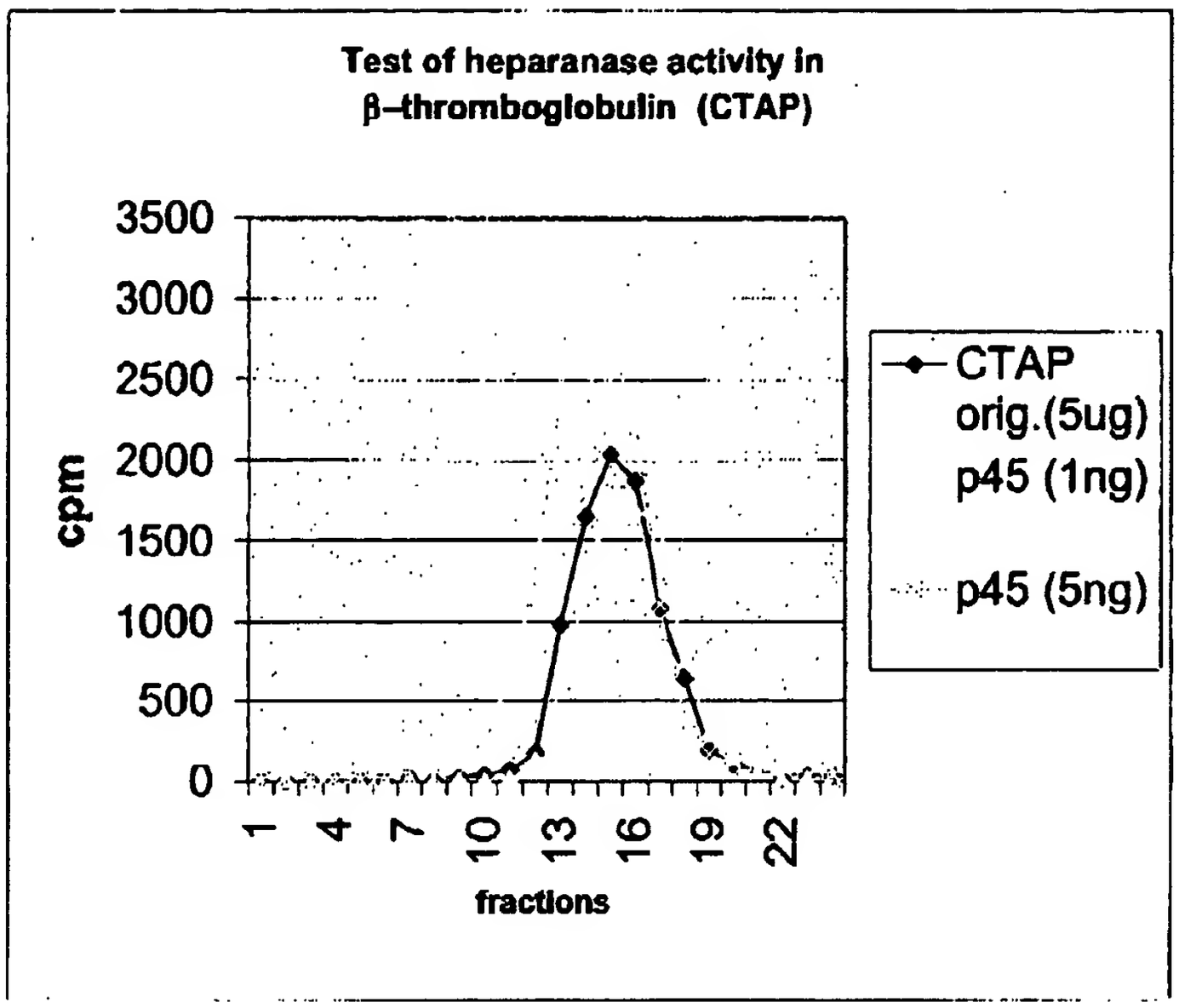

A
Heparanase
1ng
0.5ng
CTAP
5 µg
CTAP
5 µg
B
62.0 kDa
47.5 kDa
32.5 kDa
25.0 kDa
16.5 kDa
6.5 kDa

FIGURE 2


Test of heparanase activity in
β–thromboglobulin (CTAP)
3500
3000
2500
2000
1500
1000
500
0
cpm
1
4
7
10
13
16
19
22
fractions
CTAP orig.(5ug)
p45 (1ng)
p45 (5ng)

Thus, it is clear that the antibodies according to the present invention are novel and are also inventive over the background art, none of which taught or suggested a truly specific molecular probe against heparanase.

I hereby certify that the above facts and statements are true and complete, to the best of my knowledge.

Dr. Iris Pecker

Date: 9/6/03

Appendix A

The proteins identified by the inventors following the performance of Mono-S HPLC purification were found to be as follows: PAI-I, Nexin-I, Vimentin, Grp94/endoplasmin, FLT receptor, Tryptase.

Appendix B

The following is a list of published proteins which were once erroneously thought to be "pure" heparanase:

1. PAI-1 - antibodies generated against purified heparanase by Fuks & Vlodavsky (inventors of US Patent No. 5,362,641) were found to detect PAI-1 rather than heparanase (Vlodavsky, personal)

2. CTAPIII -Hoogewerf et al. J Biol Chem 1995 Feb 17;270(7):3268-77

3. GRP94/endoplasmin (De Vouge et al. Int J Cancer 1994 Jan 15;56(2):286-94)

Appendix C

Peptides identified as *nexin*-I: (Vlodavsky, personal)

TFVAADGK

SENLHVSHILQK

SYQVPMLAQLSVFR

XGSTSAPNDLXYNFIE(?)XPY

LVLVNAVYFK

HNPTGAVLFM?XQI

Unindentified peptides: (Vlodavsky, personal)

XYGPDVGQPR

QVFFEAG?NYH?LVDENE

GLSPAYLR

XATDED(Y/L)(T/L)N(P/A)DV

VAASIYT

S?VQLF?(S/G)N(T/K)

SFLK

LLR

(not listed: sequences for peptides of the other identified proteins)

Appendix D

Expression of heparanase in E.coli, which is the easiest expression system and which would be the obvious choice for a person skilled in the art, results in unfolded or misfolded polypeptide which appears in inclusion bodies and which is obviously inactive. Yeast cannot produce active heparanase because no processing of pro-heparanase occurs in any microbial system, including that of yeast. Expression of full length heparanase cDNA in yeast results in secretion of a latent preproheparanase, which has no measurable activity (see for example US Patent No. 6348344: Genetically modified cells and methods for expressing recombinant heparanase and methods of purifying same).

Appendix E

Insect cells are not a typical choice for protein expression, since post-translational processing for mammalian proteins is typically not performed properly in insect cells. For the present invention, heparanase activity was detected due to a minor proteolytic activity of an endogenous insect cell non-specific protease. This resulted in a small fraction of partially processed active heparanase which could be detected due to the use of the most sensitive heparanase ECM assay. However, as described above, such a higher level of activity would be expected from a recombinant enzyme.

Appendix F

It should be noted that the exact, complete structure of heparanase is not currently known. It has been shown that mature active heparanase contains two subunits 45kDa and 8 kDa. The interaction is non-covalent and does not involve S-S bridges. There is no information regarding the location or chronology of processing events, although it has been shown that these two subunits are separated by another peptide in the pre-pro form of heparanase, in which the two subunits and the third peptide together form a single long peptide.